Characterization of Labeled Oligonucleotides Using Enzymatic Digestion and Tandem Mass Spectrometry

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A simple and powerful method for the determination of labeling sites on oligodeoxynucleotides (ODN) has been developed. The method is based on the finding that nuclease P1 (NP1) digestions of label-containing ODNs produce site-specific products: 5′-labeled ODNs produce label–nucleotide (L–N); 3′-labeled ODN produces phosphorylated label (pL); and a label in between the ODN termini produces pL–N. Mass spectrometry spectra of these products from the digestion mixture can be easily utilized for structural verification of labeled ODNs such as DNA probes. We also developed a method for the determination of the labeling sites of ODNs with unknown label structures. In this method, NP1 digestion products generate site-specific fragmentation patterns upon collision-induced dissociation. These patterns can be easily recognized and used for the identification of labeling sites of ODNs with unknown label structures. When an ODN is internally labeled, phosphodiesterase digestion may be used to determine the exact labeling site (sequence location). It was demonstrated that these methods can be applied for ODNs with single or multiple labels, and for ODNs with the same or different labels within an ODN. (J Am Soc Mass Spectrom 1998, 9, 660–667) © 1998 American Society for Mass Spectrometry

The diverse modifications of oligodeoxynucleotides (ODN) for diagnostic research present unique challenges to the analytical chemist. These modifications often include the incorporation of chemical labels; e.g., phosphate, amine, thiol, biotin or fluorescein, at the 5′ or 3′ terminus of a DNA probe (labeled ODN), for the purpose of detection, coupling, or immobilization [1]. Although the synthesis of ODNs has become routine in many laboratories, the analytical methods have been limited to chromatographic and electrophoretic techniques that usually provide purity information only. Further characterization of DNA probes in the diagnostics industry includes base composition analysis [2]. This technique is based on high-performance liquid chromatography (HPLC) analysis of the enzymatic digestion products, however, the results usually cannot specify label location(s), nor can they distinguish different labels definitely.

The introduction of electrospray (ES) [3] and matrix-assisted laser desorption/ionization (MALDI) [4] mass spectrometry (MS) in the late 1980s made it possible to analyze large ODNs. These new mass spectrometry techniques have become powerful tools for the determination of both accurate molecular weight (MW) and chemical structures of ODNs [5–8]. Characterization of ODNs often includes the determination/verification of base sequences and the identification of base modifications. Sequence analysis can be achieved by direct mass spectrometry method using gas-phase fragmentation of the pseudo-molecular ions of ODNs such as desorption/ionization induced fragmentation, tandem mass spectrometry, nozzle–skimmer, and photodissociation [5]. Sequencing ODNs can also be achieved by indirect methods by mass spectrometry analysis of enzymatic digestion products of the ODNs [5]. Exonucleases (5′- or 3′-phosphodiesterase) have been used the most for sequence analysis [9]; base-specific endonuclease such as RNase T1 has been used for sequence location of post-transcriptional modifications of RNAs [10]; an acid hydrolysis approach [11] was also proposed to characterize ODNs, but no application has been reported. For structural analyses of modified DNAs with modified bases or DNA-carcinogen adducts, there have been extensive studies using MS/MS analysis of enzymatic digestion products [12–15]. Direct characterization of ODNs with modified bases using MS/MS or desorption/ionization induced fragmentation has also been reported [16–21].

Although regular and base-modified ODNs have been studied by mass spectrometry techniques, characterization of the labeled ODNs, with labels incorporated into ODN backbone by regular phosphodiester bonds

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through the phosphoramidite approach, has not been reported. We report here two mass spectrometry methods for the characterization of such labeled ODNs. Enzymatic digestion products of ODNs are analyzed using ionspray mass spectrometry (MS) and tandem mass spectrometry (MS/MS). Results from these experiments permit the determination/verification of ODN labeling sites and the verification of label structures.

Experimental

Mass Spectrometry

An API III+triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) was used for all experiments. The instrument has a mass-to-charge ratio range of 0–2400 and is fitted with a pneumatically assisted electrospray (ionspray) interface. Multiply charged ODN ions were generated by spraying the solution through a stainless steel capillary held at −4000 V. The sample solution was delivered to the sprayer by a syringe infusion pump (model 22, Harvard Apparatus, South Natick, MA) through a fused silica capillary of 100-μm inner diameter. The carrier solvent was 1:1 acetonitrile/water with 2 mM NH_4HCO_3. The carrier flow rate was set at 20 μL/min for sample introduction. ODN samples were dissolved in or diluted with 1:1 ACN/20 mM NH_4HCO_3. The potential on the sampling orifice was set at 35 V during calibration and was changed to −65 V for DNA signal enhancement. The instrument mass-to-change ratio scale was calibrated with the singly charged ammonium adduct ions of poly(propylene glycols) (PPGs) under unit resolution (50% valley definition). For collision-induced dissociation (CID) tandem mass spectrometry (MS/MS), collision gas thickness is set at 250 (2.5×10^{14} Ar atoms/cm^2). Nebulization and curtain gases are set at 0.6 L/min.

Chemicals and Enzymatic Digestions

Chemicals. All ODNs were synthesized in-house on a Applied Biosystems 380B DNA synthesizer using 2-cyanoethylphosphoramidite coupling chemistry and were purified by reverse-phase high performance liquid chromatography (RP-HPLC). Phosphoramidites, including biotin and fluoroscein phosphoramidites were purchased from Clontech Laboratories (Palo Alto, CA) or prepared in-house. Bacterial alkaline phosphatase (BAP), phosphodiesterases (PDEs), nuclease P1 (NP1), and nuclease S1 (NS1) were purchased from Boerhinger Mannheim Biochemicals (Indianapolis, IN). All solvents are HPLC grade or better and were purchased from EM Science (Gibbstown, NJ).

Purification of enzymes. To ensure best performance of enzymes and mass spectrometry analysis, all enzymes were purified with Microcon 50 microconcentrators (Amicon, Beverly, MA). For snake venom phosphodiesterase (3′-PDE), which was suspended in concentrated $(NH_4)_2SO_4$ when purchased, 40 μL was centrifuged to pellet the enzyme. The pellet was re-dissolved in 80 μL distilled water, and the solution was loaded to a Microcon 50. The filters were washed three times with 80 μL of distilled water and reconstituted in distilled H_2O to a desired concentration. For calf spleen phosphodiesterase (5′-PDE), which was dissolved in 50% glycerol, a similar procedure was used except that no centrifugation was performed. BAP was purified the same way as 3′-PDE. Only NP1 was dissolved in distilled water without further purification.

3′-PDE digestion. Ten μL of 1/1 acetonitrile/2 mM NH_4HCO_3 and 1–2 μL of 2 units/mL of the enzyme were added to 0.02 OD units of ODN (~100 pmol) sample. The mixture was incubated in a 37 °C water bath for 30 min.

5′-PDE digestion. Ten μL of 20 mM $HCOONH_4$ (pH 6.6) and 1–2 μL of 2 units/mL of the enzyme were added to 0.02 OD units of ODN sample. The mixture was incubated in a 37 °C water bath for 30 min.

NP1 digestion. Ten μL of 20 mM $HCOONH_4$ aqueous solution and 0.1 unit of NP1 were added to 0.02 OD units of ODN sample. The reaction mixture was incubated in a 50 °C water bath for 20 min.

NS1 digestion. Ten μL of 20 mM $HCOONH_4$ aqueous solution and 1 unit NS1 were added to 0.02 OD units of ODN sample. The reaction mixture was incubated in a 50 °C water bath for 20 min.

BAP digestion. Ten μL of 10 mM NH_4HCO_3 (or 1:1 ACN/H_2O+2 mM NH_4HCO_3) and 0.2 units of BAP were added to 0.02 OD units of DNA sample. The mixture was incubated for 20 min in a 37 °C water bath before mass spectrometry analysis.

Results and Discussion

Mass Spectrometry Analysis of NP1 Digestion Products for the Determination of ODN Labeling Sites with Known Label Structures

DNA probes are frequently labeled at various locations along their ODN backbones. Such labels include biotin, fluoroscein, rhodamine, dansyl, carbazole, and adamantane, that are incorporated into ODN backbone via phosphoramidite chemistry in automated DNA synthesis. Because biotin (B) is among the most widely used nonradioactive DNA labels that can be detected by a secondary detection system such as a biotin–enzyme–conjugated-streptavidin system [16], five biotin-containing model ODNs were synthesized and studied. These model ODNs contain the same base sequence and differ only in the locations of their biotin moiety, as shown in Table 1. ODNs I, III, and II are of the same MWs, with biotin at 5′-terminus, 3′-terminus, and in

Table 1. Model ODNs containing biotin moieties

Sequence	ODN #
5′-**B** CGT ATG AGT GAT TCC TCC-3′	I
5′-CGT ATG AGT **B** GAT TCC TCC-3′	II
5′-CGT ATG AGT GAT TCC TCC **B**-3′	III
5′-CGT ATG AGT GAT TCC TCC **Bl**-3′	IV
5′-**B** CGT ATG AGT **B** GAT TCC TCC **Bl**-3′	V

between the termini, respectively. ODN IV contains a biotin at the 3′-terminus and an additional short universal linker [OH–$(CH_2)_2$–OH] on the biotin moiety (**Bl**). ODN V contains three biotins, each located at the same site as in one of the above three ODNs (I, II, and IV). The structures of these probes are shown in Figure 1.

Various nucleases have been used to digest these model ODNs and the digestion products were analyzed by electrospray mass spectrometry. These enzymes included NP1, NS1, 3′-PDE, and 5′-PDE. Digestion products from using all of these enzymes provided some information about the labeling sites, with NP1 giving the most complete information and the highest digestion efficiency. NS1 digestion provided similar information, but with slower digestion rate and relatively poor digestion efficiency. PDE digestions provided some information, but did not work in many cases.

PDE digestions are known to require a free 3′-terminus (3′-PDE) or 5′-terminus (5′-PDE). Using the model ODNs, it was found that such digestions stopped at one base away from the label from the direction of digestion. As a result, mass spectrometry analysis of PDE digestion products provided only limited information of labeling sites, as shown in Table 2. With 3′-PDE, a biotin-labeled nucleotide **B**–C was produced following the digestion of ODN I. An ion corresponding to **B**–C was observed by mass spectrometry. With ODN II, 5′-CGT ATG AGT **B** G was generated following the digestion, and the detection of such a shortened ODN indicated that there is at least one label in the middle of the ODN backbone. For ODN III, IV, and V, no 3′-PDE digestion products were found, indicating a label at the 3′-terminus. When 5′-PDE digestions were performed for the five above mentioned compounds, more information was obtained, as shown in Table 2. However, no information was obtained when both termini are labeled (DNA V).

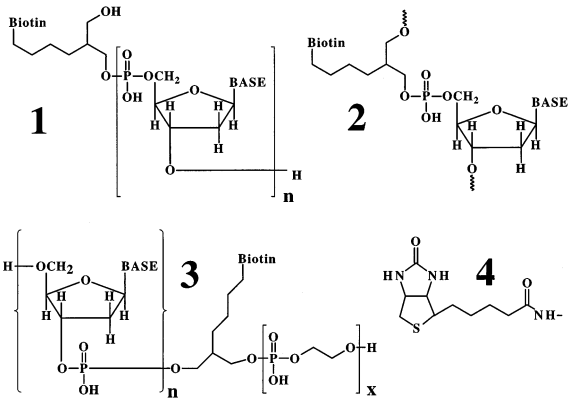

Figure 1. The structures of labeled ODNs: (**1**) 5′-biotinylated ODNs; (**2**) ODNs with biotin in-between termini; (**3**) 3′-biotinylated ODNs with ($x = 1$) and without ($x = 0$) the universal linker; (**4**) biotin moiety in labeled ODNs. Squiggly lines in the structures represent the extension sites of ODN backbone structures.

Nuclease P1 digestion, on the other hand, provides much more information about labeling sites, regardless of the locations of the labels. Figure 2a–e shows the mass spectrometry spectra of NP1 digestion products of the five model oligonucleotides, which are also shown in Table 2. In the low mass-to-charge ratio range of all mass spectra, four major ions at m/z 306.2, 321.2, 330.2, and 346.2, are always observed. These ions correspond to deprotonated molecules of the four nucleosides 5′-monophosphate (5′-NMP), which are standard NP1 digestion products of regular ODNs. In addition to these standard ions, site-specific ions corresponding to the three biotin locations in the five model ODNs (Figure 2a–e) are observed.

With the biotin label at the 5′-terminus (ODN I), the characteristic ion observed is m/z 661.2 (Figure 2a). MW calculation of fragments at all potential cleavage sites indicates that this ion corresponds to deprotonated biotin–nucleotide (**B**–N). Similarly, a different ion at m/z 781.2 (Figure 2b) is observed for the ODN with a biotin in-between the termini (ODN II). Out of the same consideration, the digestion product is proposed to be a phosphorylated biotin–nucleotide (p**B**–N), very similar to the product from 5′-biotin labeled ODN. With the biotin label at the 3′-terminus (ODN III), the characteristic ion is found to be m/z 452.2 (Figure 2c). The corresponding digestion product is proposed to be p**B**, a phosphorylated biotin moiety. When a universal linker is attached to the 3′-terminal biotin (ODN IV), the characteristic ion is found to be m/z 576.2 (Figure 2d). The corresponding digestion product is proposed to be p**Bl**, a phosphorylated biotin moiety with the linker attached. A comparison between NP1 digestion of ODN III & IV indicates that the phosphodiester bond between

Table 2. Proposed digestion products as observed by mass spectrometry

Enzyme\ODN	ODN I	ODN II	ODN III/IV	ODN V
5′-PDE	None	T **B** GAT TCC TCC-3′	C–**B**/C–**Bl**	None
3′-PDE	**B**–C	5′-CGT ATG AGT **B** G	None	None
NP1	**B**–C	p**B**–G	C–**B**/C–**Bl**	**B**–C & p**B**–G & C–**Bl**

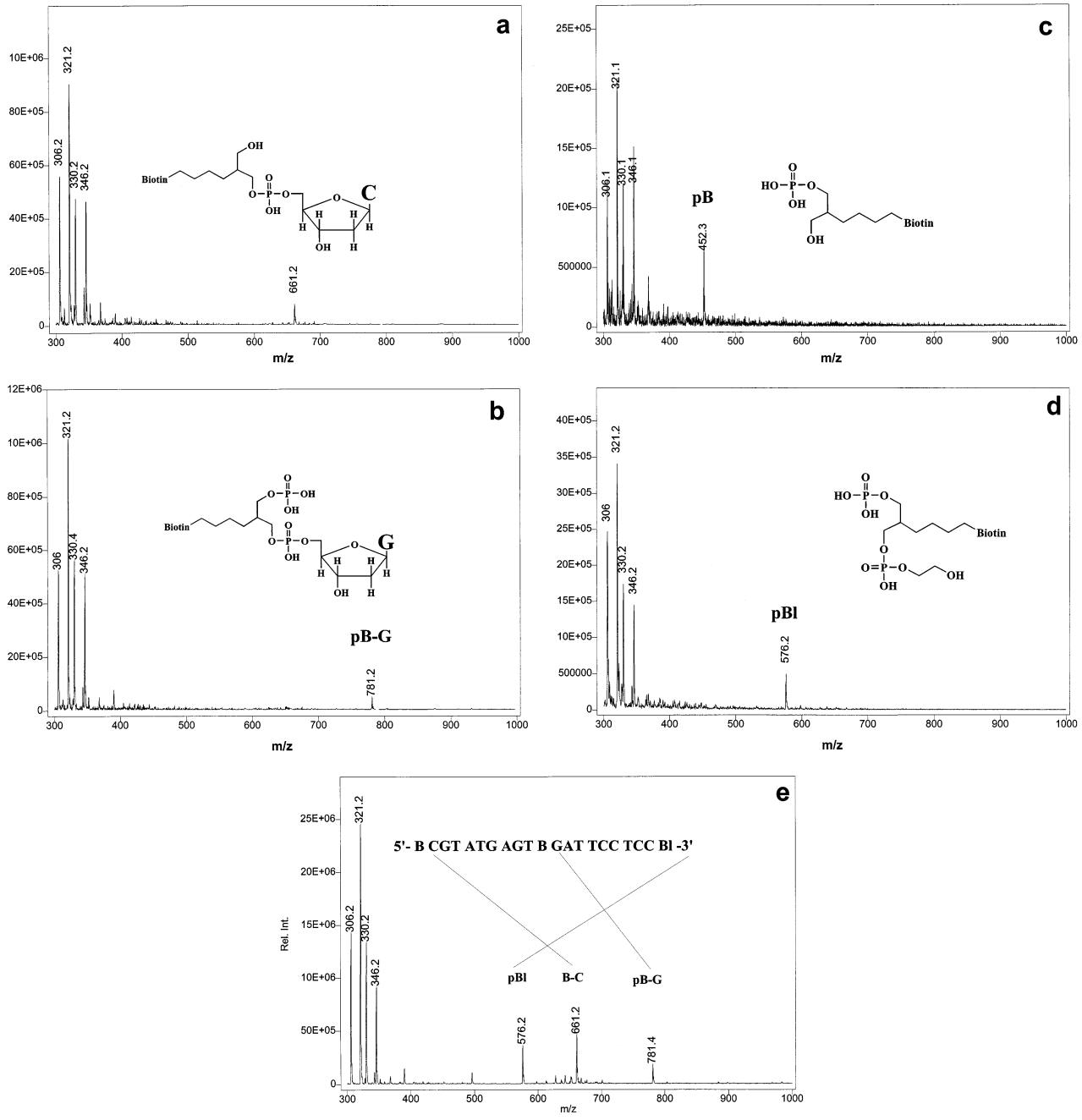

Figure 2. Mass spectrometry spectra of NP1 digestion products of five model ODNs: (**a**) 5′-**B** CGT ATG AGT GAT TCC TCC-3′; (**b**) 5′-CGT ATG AGT **B** GAT TCC TCC-3′; (**c**) 5′-CGT ATG AGT GAT TCC TCC **B**-3′; (**d**) 5′-CGT ATG AGT GAT TCC TCC **Bl**-3′; (**e**) 5′-**B** CGT ATG AGT **B** GAT TCC TCC **Bl**-3′.

the biotin moiety and the universal linker cannot be cleaved by the NP1.

The above results indicate that NP1 digestion of ODNs with biotin(s) at different sites generates site-specific products. ODNs with biotins at the 5′-terminus, 3′-terminus, and in between form B–N, pB, and pB–N, respectively, and the site-specific ions from these digestion products on a mass spectrum can be used to determine the location(s) of biotin along the ODN backbone.

To test the method on more complex samples, ODN V containing biotins at all three locations was digested using NP1 and mass spectrometry analyzed. All of the three characteristic ions were observed (Figure 2e), showing a biotin moiety at all three different sites. This result indicated that this simple method can be used to identify multiple labels on an ODN. In a single digestion, the biotin labels at all three different locations were identified due to their site-specific products, demonstrating the effectiveness of this simple technique.

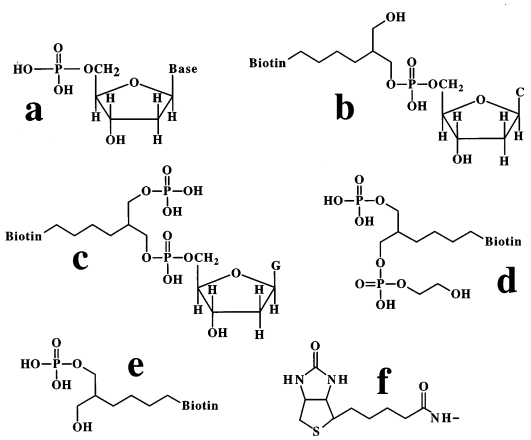

Figure 3. Chemical structures of NP1 digestion products of the five model ODNs: (**a**) 5′-NMP; (**b**) B–C; (**c**) pB–G; (**d**) pBl; (**e**) pB; (**f**) the structure of the biotin moiety.

The structures of these products, as shown in Figure 3, were characterized and confirmed by CID MS/MS. The CID-MS/MS spectra are shown in Figure 4a–d. The product ions observed are consistent with the proposed structures for these products.

Based on the above observations, the final site-specific products from a NP1 digestion of an ODN can be easily predicted when its sequence and label structure are provided. For ODNs with the same biotin label structures as in the five model ODNs, biotin locations can be obtained from NP1 digestion and mass spectrometry analysis. Table 3 shows the characteristic ions from such ODNs. It should be pointed out there is no confusion in assigning the locations of biotin labels because the structures of these digestion products are so distinctively different: B–N, pB–N, and pB/pBl. For instance, the heaviest B–N ion is at m/z 701.2 (B–G) and the lightest pB–N is at m/z 741.2 (pB–C).

The universality of the method was also studied using other common labels (L) such as fluorescein, dansyl, carbazole, and adamantane, that are joined to the ODNs by regular phosphodiester bonds through the phosphoramidite approach. It was found that NP1 digestion of ODNs containing these labels generated products with the same patterns as those for biotinylated ODNs; and the digestion products are shown in Table 4.

When an ODN is modified by distinct labels, all the labels can be identified effectively with this method in a single digestion. Figure 5 shows the mass spectrometry spectrum of the NP1 digestion products of a DNA probe containing both biotin and carbazole. The locations of both labels were clearly identified in a single spectrum.

With labeled homo oligonucleotides, caution must be taken when interpreting the mass spectrometry spectrum. This is clearly demonstrated with 5′-fluorescein-A_{25} (Figure 6a), an additional peak at m/z 661.2 was observed; and it can be easily misinterpreted as a peak derived from a different label. However, the CID-MS/MS spectrum (Figure 6b) of the ion indicates that it corresponds to cluster ions $(n\text{M–H})^-$, with $n = 2$ and M = AMP (adenosine 5′-monophosphate). The cluster formation is also evidenced by the presence of a very intense peak of 5′-AMP at m/z 330.2, absence of other nucleoside 5′-monophosphates (5′-NMP), and the cluster ions at m/z 992.3 ($n = 3$). The formation of the cluster ions is assumed to be the result of the relatively high concentration of 5′-AMP in the digestion mixture.

As has been demonstrated above, this enzymatic method followed by mass spectrometry analysis can be readily applied for structure verification of labeled ODNs such as DNA probes. This makes it a powerful technique for quality control of labeled ODNs.

MS/MS Analysis of NP1 Digestion Products for the Determination of ODN Labeling Sites with Unknown Label Structures

When ODNs contain unknown labels that vary in label structure and linker length, it is difficult to apply the above discussed method for the identification of labeling sites based on MW determination of the digestion products. To solve this problem, we studied the above NP1 digestion products using CID-MS/MS. The MS/MS spectra of these digestion products provide distinctive features that can be applied for the determination of the locations of labels on ODNs.

For the digestion products (B–C) from the 5′-labeled ODN I, the MS/MS spectrum (Figure 4a) shows the following major peaks: $(\text{M–H–base})^-$ ion (m/z 550) from loss of the base (cytosine) and $(\text{M–H–nucleoside})^-$ ion (m/z 452) from loss of the nucleoside. Loss of 5′-NMP to produce $(\text{M–H–NMP})^-$ ions (m/z 354) cannot be detected presumably because the biotin moiety does not contain functional groups that can stabilize negative charges effectively.

With pB–G, a digestion product from ODN II & V, major fragment ions (Figure 4b) are totally different from those observed for the dephosphorylated analog (Figure 4a). In fact, the major fragment ions for B–C, $(\text{M–H–base})^-$ and $(\text{M–H–nucleoside})^-$, are no longer major ions. Instead, three other ions become dominant: $(\text{M–H–NMP})^-$ ions from loss of 5′-NMP (m/z 434.2), $(\text{M–H–}H_3PO_4)^-$ ions from loss of H_3PO_4 (m/z 683) from the molecular ion $(\text{M–H})^-$, and $(\text{5′-NMP–H})^-$ from loss of the label moiety (m/z 346.0). To find out if the MS/MS spectral difference between B–C and pB–G is because of the base difference, pB–G was dephosphorylated by bacterial alkaline phosphatase (BAP). The resulting product B–G was subjected to CID-MS/MS (Figure 4c) and the same pattern as that for B–C (Figure 4a) was observed. Therefore, the dramatic spectral difference between B–C and pB–G (Figure 4a, b) is not the result of base difference but the consequence of phosphorylation. The additional phosphate group adds two more favorable deprotonation sites from which different fragment ions are formed.

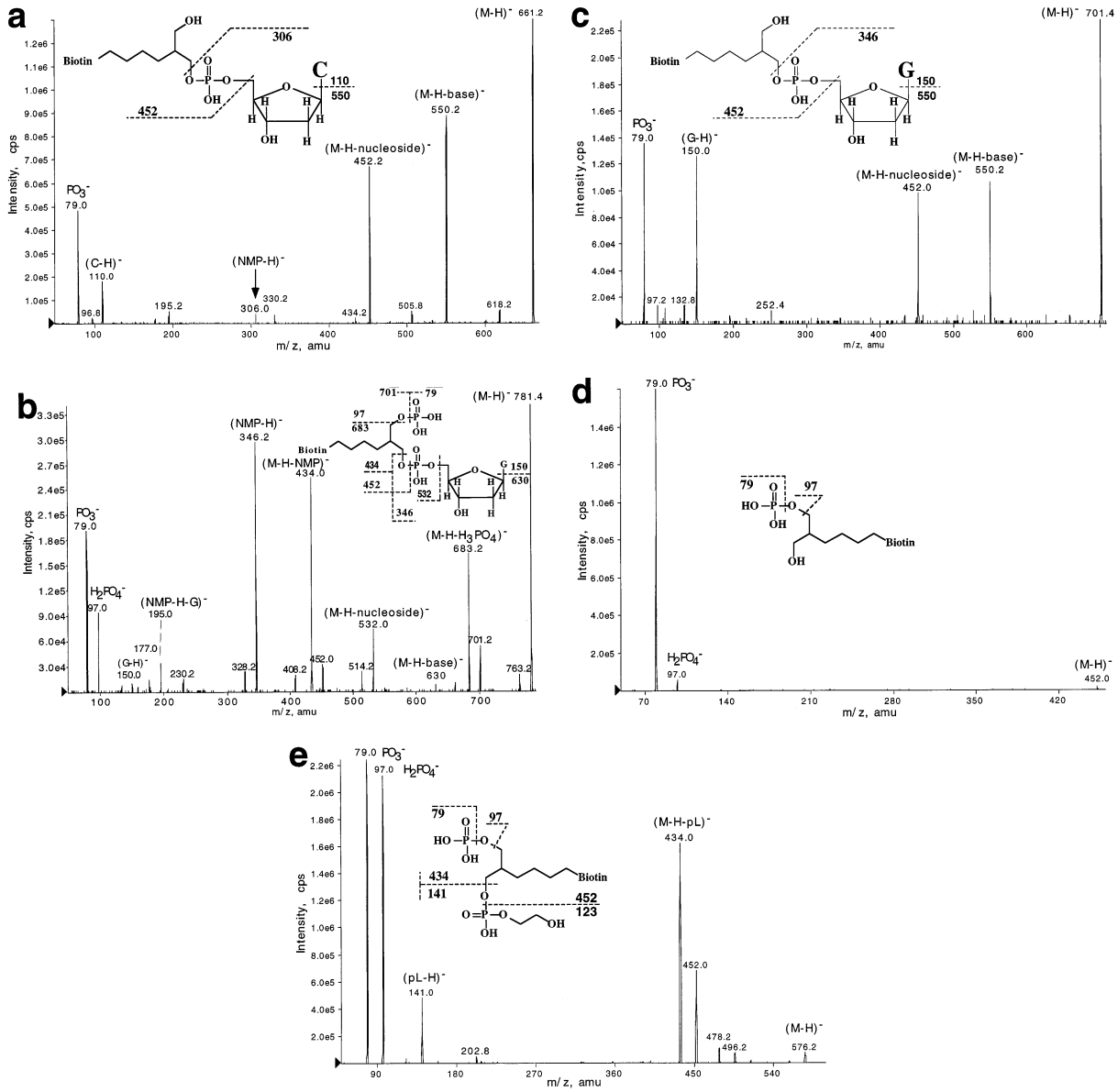

Figure 4. CID-MS/MS spectra of NP1 digestion products of the model ODNs: (**a**) m/z 661.2 of B–C; (**b**) m/z 781.2 of pB–G; (**c**) m/z 701.2 of B–G; (**d**) m/z 452 of pB; (**e**) m/z 576 of pBl. L in (**e**) designates the linker $HOCH_2CH_2OH$.

Table 3. Biotin locations and the end NP1 digestion products observed by mass spectrometr

5′-Biotin				
Products in general	**B**iotin–nucleotide (**B**–N)			
Possible products	B–C	B–T	B–A	B–G
Ions [m/z of $(M–H)^-$]	661.2	676.2	685.2	701.2
Biotin in between the termini				
Products in general	pB–N			
Possible products	pB–C	pB–T	pB–A	pB–G
Ions [m/z of $(M–H)^-$]	741.2	756.2	765.2	781.2
3′-Biotin (or 3′-biotin-linker)				
Product	pB or pBl			
Ions [m/z of $(M–H)^-$]	452.2 for pB (or 576.2 for pBl)			

Table 4. NP1 digestion products of ODN containing phosphodiester-bond linked labels

Labeling sites	Digestion products
5′-Label	L–C, L–T, L–A, L–G
Label in between the termini	pL–C, pL–T, pL–A, pL–G
3′-Label	pL

Comparison of the above MS/MS spectra clearly shows that major fragment ions for B–N are minor for pB–N, and vice versa. This is clearly shown in Table 5. The MS/MS spectral difference is presumably because of the fact that negative charges for all the ions formed reside favorably on the phosphate group or the phosphodiester linkage because of their high gas phase acidities, and that charges at other sites are not as stable. This is especially true when the label moiety such as biotin/fluoroscein contains no site that can be deprotonated as easily as the phosphate group and the phosphodiester linkage.

With pB, a NP1 digestion product of the 3′-labeled ODN III, a dominant fragment ion at m/z 79 (PO_3^-) is found (Figure 4d). All other ions become insignificant. For the pBl, dominant ions include phosphate group and linker related ions (Figure 4e): PO_3^- ions at m/z 79, $H_2PO_4^-$ ions at m/z 97, deprotonated phosphorylated linker (pL) ions at m/z 141, loss of pL at m/z 434. The dominance of these ions (m/z 79, m/z 97) and the absence of nucleotide bases can be used as the indication for labels at the 3′-terminus.

Clearly, the MS/MS spectra of the digestion products from labeled ODNs have shown site-specific fragmentation patterns, as summarized in Table 5. These patterns can be readily used to determine if a digestion product under investigation contains a label at the 5′- or 3′-terminus or in between the two termini. When ODN samples contain unknown labels, the MS/MS spectra of the digestion products can be obtained first. If a site-specific digestion product (M) contains a nucleotide base as in the case of ODNs with labels at the 5′-terminus (L–N) or in between the termini (pL–N), the base bonded to the label can be readily identified by the presence of the deprotonated base (purines and pyrimi-

Table 5. CID-MS/MS ion abundance of digestion products

	Ion abundance[b]			
Fragment ions[a]	B–G	pB–G	pB	pBl
PO_3^- (m/z 79)	m	m	**hh**	**h**
$(Base–H)^-$ (m/z 110, 125, 134, 150)	m	m		
$(NMP–H)^-$ (m/z 306, 321, 330, 346)	l	**h**		
$(M–H–H_3PO_4)^-$	l	**h**		
$(M–H–base)^-$ (M–112/127/136/152)	**h**	l		
$(M–H–nucleoside)^-$	**h**	l		
$(M–H–NMP)^-$	l	**h**		
Phosphorylated linker [$(pL–H)^-$]				h
$(M–H–pL)^-$				h
$H_2PO_4^-$ (m/z 97)	l	m	w	h

[a]Abbreviations: base—nucleoside base; NMP—nucleoside 5′-monophosphate; nucleoside—dehydrated nucleosides with unknown structures (refer to spectra).
[b]Abundance: l—low, m—medium, h—high, hh—dominant; collision energy = 30 eV.

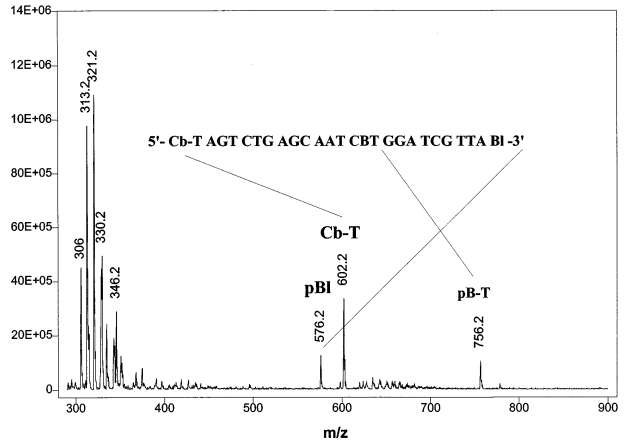

Figure 5. MS spectrum of NP1 digestion products of an ODNs labeled with carbazole (Cb) and biotin. "Bl" stands for biotin is attached to a 3′-terminal universal linker (see text for details).

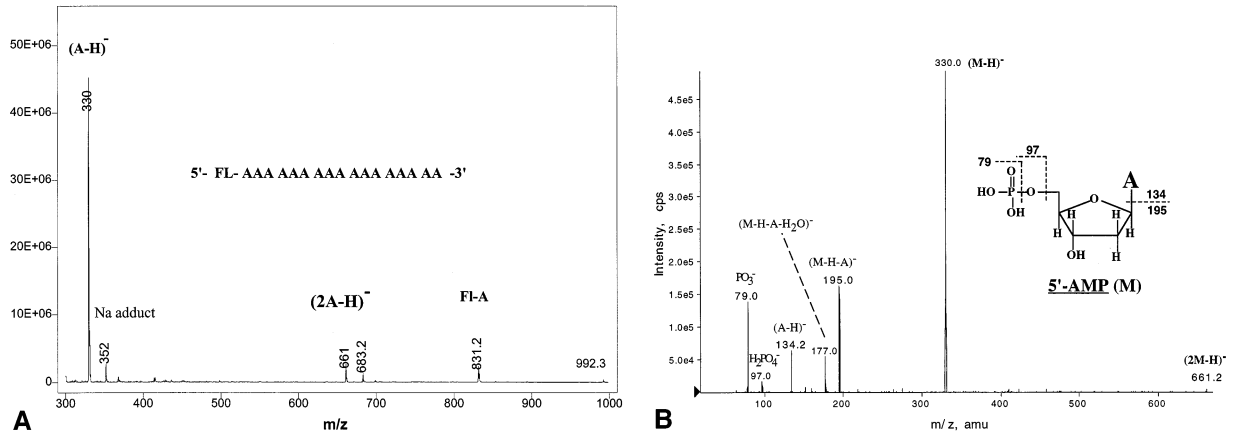

Figure 6. Mass spectrometry spectrum of fluorescein (Fl)-labeled homo ODN Fl–A25; (**b**) CID-MS/MS spectrum of m/z 661.2 of 5′-AMP dimer.

dines) at m/z 110, 125, 134, and 150 for C, T, A, & G, respectively. The presence of the bases can often be verified by the presence of $(M–H–base)^-$ or $(M–H–NMP)^-$. In contrast, absence of all these ions from MS/MS spectra of the site-specific products is an indication that the label is at the 3′-terminus. High abundance of $(NMP–H)^-$ ion or $(M–H–H_3PO_4)^-$ ion is an indication that a label is in between the termini. High abundance of $(M–H–nucleoside)^-$ and $(M–H–base)^-$ is an indication that a label is at the 5′-terminus. Dominance of m/z 79 and absence of nucleotide bases indicates a label at the 3′-terminus. It should be pointed out that the relative intensity (l, m, or h) should be compared within one spectrum and that any conclusion based on a single ion should be confirmed by other ions.

When ODNs contain other labels such as fluorescein and carbazole, the same fragmentation and relative abundance patterns have been observed (data not shown). Therefore, the fragmentation patterns of deprotonated digestion products from CID-MS/MS spectra can be used to determine the labeling sites on unknown ODNs. In practice, major ions at mass-to-charge ratio values above 400 should be analyzed by MS/MS. For 3′-labeled ODNs, dominant ions are m/z 79, 97 and ions from linker or loss of linker. For 5′-labeled ODNs, $(M–H–base)^-$ and $(M–H–nucleoside)^-$ is much more abundant than $(NMP–H)^-$, $(M–H–H_3PO_4)^-$, and $(M–H–NMP)^-$. If the observed abundance pattern is reversed, the labels must be in-between the two termini.

Conclusions

We have developed a simple and powerful method for locating labeling sites on ODNs. The method is based on the findings that NP1 digestion of label-containing ODNs produces site-specific products: a 5′-labeled ODN produces an ion corresponding to label–nucleotide (L–N); a 3′-labeled ODN produces an ion of phosphorylated label (pL); and a label in between the ODN termini produces an ion of pL–N. These digestion products can be readily identified by mass spectrometry to determine the labeling sites, and the chemical structures of the labels can be verified by the CID-MS/MS spectra of the digestion products. The effectiveness and simplicity of this method make it a valuable tool for the characterization of labeled ODNs such as DNA probes.

We also developed a method for the determination of labeling sites of ODNs with unknown label structures, based on the CID-MS/MS spectra of NP1 digestion products of the samples. CID-MS/MS of NP1 digestion products generates site-specific fragmentation patterns. These patterns can be easily recognized and used for the identification of ODN labeling sites with unknown label structures. It has been demonstrated here that both methods can be applied for ODNs with single or multiple modifications, and for ODNs with the same or different labels within an ODN.

The limitations of the methods described in this work should be pointed out. The methods with NP1 digestion can determine the exact labeling sites (sequence locations) when a label is at 5′- or 3′-terminus of an ODN. When a label is incorporated in between the termini of an ODN, however, the combination of NP1 digestion and ES-MS or ES-MS/SM can only determine that the ODN is internally labeled and to which of the four nucleotides the label is attached, but it cannot determine the exact labeling site (sequence location). In such cases, if one of the termini such as 3′-terminus is free, 3′-PDE digestion can be performed to determine the exact labeling site, as data shown in Table 2. If both termini are labeled, the exact labeling site of an internal label cannot be determined by mass spectrometry or MS/MS analysis of the NP1 digestion products. In such a case, CID-MS/MS analysis of the original ODN may provide the labeling-site information of the internal label if the ODN is relatively small (ODNs of 15 bases long may be the practical limit). For larger ODNs, new methods have to be developed.

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